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The Isolation and Partial Characterization of the Lipopolysaccharides from Rhizobium leguminosarum biovar trifolii strains TB4, TB104, and TB112

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The Isolation and Partial Characterization of the Lipopolysaccharides

from *Rhizobium leguminosarum* biovar *trifolii* strains TB4, TB104, and TB112
(TITLE)

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Jill K. Miller

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ABSTRACT

The lipopolysaccharides (LPSs) from Rhizobium leguminosarum biovar trifolii TB4 and two transposon (Tn5) symbiotic mutants, R. leguminosarum bv. trifolii TB104 and TB112, were isolated and partially characterized. Bv. trifolii TB4 is a derivative of R. leguminosarum bv. trifolii ANU843. The two mutant strains elicited incompletely developed clover nodules that exhibited low bacterial populations and very low nitrogenase activity. The LPSs were extracted using the hot phenol-water method and purified with gel filtration column chromatography using Sepharose 4B with an elution buffer consisting of EDTA and triethylamine. The hexose compositions were determined by gas chromatography of the alditol acetate derivatives. LPS components were quantitated by colorimetric assays. The LPSs of both mutants lacked O-antigen sugars and some sugar residues of the LPS core oligosaccharides. Therefore, R. leguminosarum bv. trifolii TB104 and TB112 are missing LPS I, the complete form of LPS containing the O-antigen found in the parent strain TB4. Polyacrylamide gel electrophoresis analysis verifies that the mutants are missing the O-antigen through the lack of bands in the LPS I higher molecular weight region. An immunoblot procedure using bv. trifolii ANU843 antisera confirmed these results. Thus, while the parent strain contains both LPS I (O-antigen complete) and LPS II (O-antigen incomplete), the mutants contain only LPS II.

DEDICATION

to Blair and Erin

ACKNOWLEDGEMENTS

I thank Dr. Russell W. Carlson for his guidance, assistance, and patience throughout the course of this work. Also, I thank Dr. K. Dale Noel for providing the bacterial strains used in this study.

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INTRODUCTION

Nitrogen fixation is important both ecologically and economically. In the developed countries of the world, the use of nitrogen fertilizers is both widespread and essential in meeting the agricultural needs of the population. In the underdeveloped countries of the world, the expense and unavailability of industrially produced nitrogen fertilizers make their use prohibitive. Therefore, nitrogen is a limiting factor of agricultural production throughout the world (1).

The Gram-negative bacteria of the genera Rhizobium and Bradyrhizobium are able to form nitrogen-fixing symbiotic relationships with members of the family Leguminoseae (beans, soybeans, clover, etc.). This mutually beneficial relationship provides nitrogen to the plant in the usable form of ammonia and carbon to the bacteria through the products of photosynthesis. Two benefits to be gained by using legumes as crop or pasture plants are the plant's independence of soil nitrogen and the potentially improved nitrogen-status of the soil resulting from the use of the legume (2).

The host legume root nodule is the site of biological nitrogen fixation by the Rhizobium bacteria. Effective nodule development, and consequently nitrogen fixation,

occurs through a series of intricate interactions between the symbionts. First, rhizobia, present in the soil, specifically bind to new host root hairs and elicit root hair curling. Generally, the root hairs that are invaded have not yet emerged from the developing root at the time of first bacterial contact. The presence of the bacteria causes the root hairs to curl as they develop. Secondly, the host cell wall is degraded in a pocket formed by the curled root hair and the bacteria penetrate the root hair cell wall. A tubular wall is deposited by the host between the invading rhizobia and the involuted root hair cell wall, forming an infection thread. This infection thread grows through the root hair cell wall into the root cortex. Thirdly, active cell division occurs in the root cortex forming a nodule. Then, the bacteria are released from the infection thread into the cytoplasm of these nodule cells. The bacteria remain separate within the cytoplasm by peribacteroid membranes which are derived from the infection thread. Fourthly, the bacteria differentiate into bacteroids which produce the enzyme nitrogenase. The nodules are the site where atmospheric dinitrogen is converted into ammonia (3,4,5).

The expression of both host legume and microsymbiont rhizobial genes plays a role in nodule initiation and development. In both symbionts a portion of the genome is expressed only in the symbiotic state. Genome variation may

affect the sequence of nodule development. The legume produces nodulins, nodule-specific proteins. In the rhizobia, genes located on plasmids and on the chromosome are involved in the nitrogen-fixing symbiosis. Many genes for symbiotic function, including nodulation (nod genes) and nitrogenase activity (nif genes) occur on large Sym plasmids (3,6,7,8). Early symbiotic functions encoded by the Sym plasmid include bacterial binding to the root hairs, hair curling, and host specificity. Sym plasmid genes expressed during nodule development include genes for nitrogenase polypeptides and a regulatory gene of nitrogenase activity (3). Using transposon Tn5 mutagenesis, Noel et al. (9) found that symbiosis-specific mutations occur in the chromosome as frequently as in the Sym plasmid. The chromosomal mutations predominantly affected nodule development in the intermediate stages between the initial induction of nodule growth, which is at least partially determined by plasmid genes, and the mature stage of the nodule in which nif expression yields nitrogen fixation. The findings of Vandenbosch et al. (3) suggest at least three chromosomal genes may be involved.

In several Rhizobium species, symbiotic genes have been defined by isolation of mutants with differing phenotypes. Hac$^-$ mutants lack the ability to curl host root hairs (3). In the Nod$^-$ phenotype, no nodules are formed. The bacteria fail to penetrate the root and signal the plant to initiate

root cortical cell divisions. In the Fix$^-$ phenotype, bacteria invade the root via infection threads and cause nodule development; however, the bacteria either fail to differentiate into bacteroids or differentiate into bacteroids that fail to fix nitrogen (7,9). These two types of the Fix$^-$ phenotype have been described separately. In the Sna$^-$ (symbiotic nitrogenase activity) type, the nodules delay in appearing and subsequently grow much slower than normal; bacteroid content is very low. In contrast, with the Ndv$^-$ (nodule development) type, the nodules develop well at first, but show degeneration sooner than normal. The abundant bacteroids present cannot fix nitrogen (9).

The plasmid-borne nod genes so far defined (designated nodABCDEF) are arranged in three separate operons (nodABC, nodD, and nodFE) (10). Only one gene, nodD, is expressed by Rhizobium in culture, and its gene product, along with factors secreted by the legume, induces expression of genes in nodABC and nodFE operons (10). Redmond et al discovered that flavones found in washings of undamaged clover roots induce nod gene expression. The particular flavones found are 7,4'-dihydroxyflavone and geraldone, and the natural sources of these flavones are confined to the legumes with optimal concentrations occurring near the emerging root hair zone. If these flavones are restricted to this family, the ability of Rhizobium cells to express nodulation genes in response to particular flavones may represent an adaptation

that gives the Rhizobium a competitive advantage in the legume rhizosphere (10).

In the Rhizobium-legume symbiosis, there is a considerable degree of specificity between rhizobial species and the host plant species which they infect. For example, R. japonicum specifically nodulates soybeans, R. leguminosarum biovar phaseoli specifically nodulates beans, and R. leguminosarum biovar trifolii specifically nodulates clover (11,12,13). The molecular basis of this specificity remains to be determined. Rhizobia are Gram-negative bacteria and therefore have the usual surface polysaccharides consisting of extracellular polysaccharides (EPSs), capsular polysaccharides (CPSs), and lipopolysaccharides (LPSs). All these molecules have been hypothesized to play an important role in the symbiotic infection process (5,14,15). Studies to determine the role of Rhizobium EPSs and LPSs in symbiosis continue because of the strong evidence showing that the EPSs and LPSs from other Gram-negative bacteria determine the specificity of their association with other organisms. The LPSs of Salmonella and Escherichia coli are the strain-specific antigenic determinants of these bacteria and are known to be the determining factors in the specific infection of a host (16).

Lectins (phytohaemagglutinins) are a group of proteins, widely distributed in legumes, which have the ability to bind sugars and polysaccharides specifically in a manner

analogous to the antibody-antigen reaction (11,12). Several binding studies indicate that the specificity in the legume-rhizobial symbiosis might be based on the interaction between leguminous lectins and rhizobial polysaccharides (11,13,17,18). Other studies do not support this lectin-binding hypothesis (12,19). Both the EPS and LPS of Rhizobium have been postulated to play a role in determining the specificity of Rhizobium-legume symbioses (16). Several reports present data indicating that the lectin receptor on the Rhizobium is the EPS, while other reports indicate that LPS is the lectin receptor (5,14,15,16). Based on data from chemical composition studies of EPSs and LPSs, and serological and bacteriophage studies, it seems likely that several types of Rhizobium exist. The surface molecule(s) which determines symbiotic specificity could vary depending on the Rhizobium type. Data suggest that the EPSs determine symbiotic specificity in the case of the slow-growing B. japonicum. Conversely, the LPSs may determine the symbiotic specificity in the case of the fast-growing R. leguminosarum bv. leguminosarum, bv. trifolii, bv. phaseoli, and R. meliloti (16).

The LPSs have been the least studied of the Rhizobium polysaccharides. LPS is found on the outer membrane of Gram-negative bacteria. The lipid A portion anchors the LPS to the outer membrane, while the polysaccharide portion extends into the cell surroundings (20). Composition

studies show that Rhizobium LPSs vary greatly among different species as well as among strains of a single species (5). To understand the role(s) of LPSs in the rhizobial symbiotic process, it is first necessary to structually characterize these molecules. By comparing studies on the LPSs of E. coli and Salmonella to studies on the LPSs of Rhizobium species, a structural arrangement for Rhizobium LPS can be proposed. The general structure of LPSs from S. typhimurium and E. coli is shown in Figure 1 (21). These LPSs have been shown to be present in two general forms. One form, LPS I, is a complete molecule consisting of the lipid-A core oligosaccharide, and repeating oligosaccharide known as the O-antigen. The other form, LPS II, is the incomplete molecule which lacks the O-antigen. The polysaccharide portion of the LPS is attached to the lipid-A via an acid-labile ketosidic bond with 2-keto-3-deoxyoctonic acid (KDO). Mild acid hydrolysis releases a polysaccharide consisting of the O-antigen attached to the core region and an oligosaccharide which is the core region only. In addition, polyacrylamide gel electrophoresis (PAGE) studies have shown a multiple banding pattern indicating that the LPS molecules are very heterogeneous. The many bands are apparently separated from one another by a single O-antigen repeating unit (5,15).

Rhizobium LPSs have some of the same features as E. coli and Salmonella LPSs, but the general structure differs.

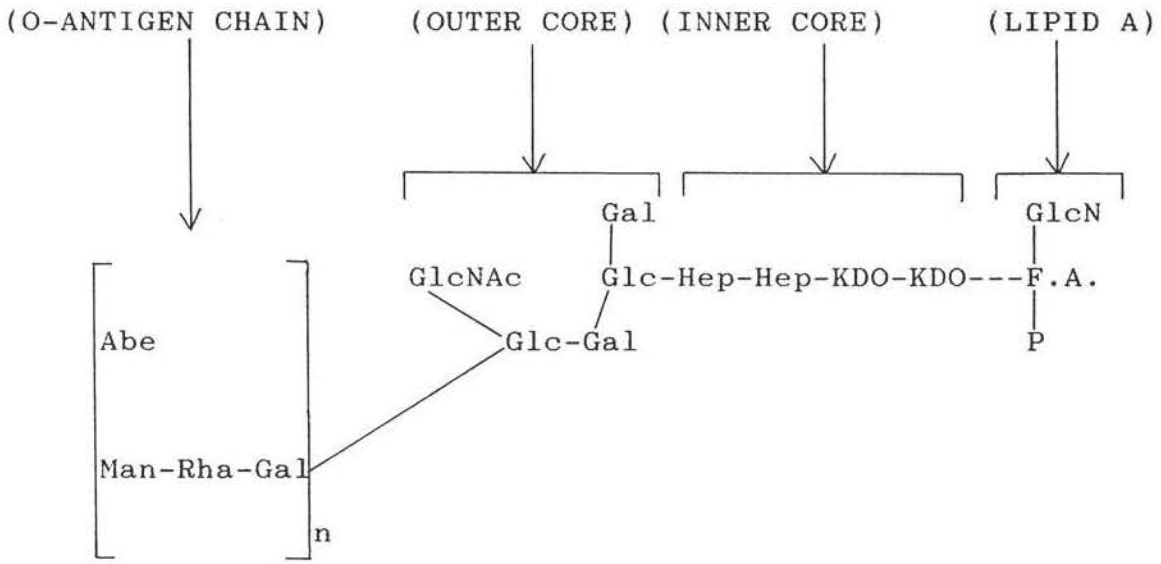

Figure 1. The general structural arrangement of the LPS components for S. typhimurium. Abe, Abequose; F.A., fatty acid; P, phosphate.

Unlike E. coli and Salmonella LPSs, mild acid hydrolysis releases a polysaccharide (PS1) and an oligosaccharide (OS; PS2) from lipid A. The PS1 polysaccharide has been termed the O-antigen and varies in composition from strain to strain. The PS2 OSs, termed the LPS core, exhibit conserved composition. The PS2s of R. leguminosarum bv.leguminosarum, bv.phaseoli, and bv. trifolii migrate similarly on gel filtration and are composed largely of galacturonic acid, with smaller amounts of mannose, galactose, glucose, and KDO. Figure 2 shows a proposed structural arrangement of Rhizobium LPS for these biovars. Carlson et al (5) have determined the structure of the major core component from a bv. trifolii LPS. It consists of two terminal galacturonic acid residues α-linked to positions 4 and 7 of KDO. Another core component of this LPS contains mannose, galactose, KDO, and galacturonic acid in a 1/1/1/1 ratio. The mannosyl residue is α-linked to position 4 of KDO, while the galactosyl and galacturonosyl residues are α-linked to the 4 and 6 positions of the mannosyl residue, respectively (Carlson, personal communication). Usually the rhizobial O-antigens are likely to be complex OSs rather than a polysaccharide formed by the polymerization of a repeating OS. Also, these O-antigens commonly contain methylated sugars and methylated amino sugars, and when heptose is present, it is found in the O-antigen and not in the core OS. For several bv. trifolii LPSs, both the O-antigen and core have KDO at their

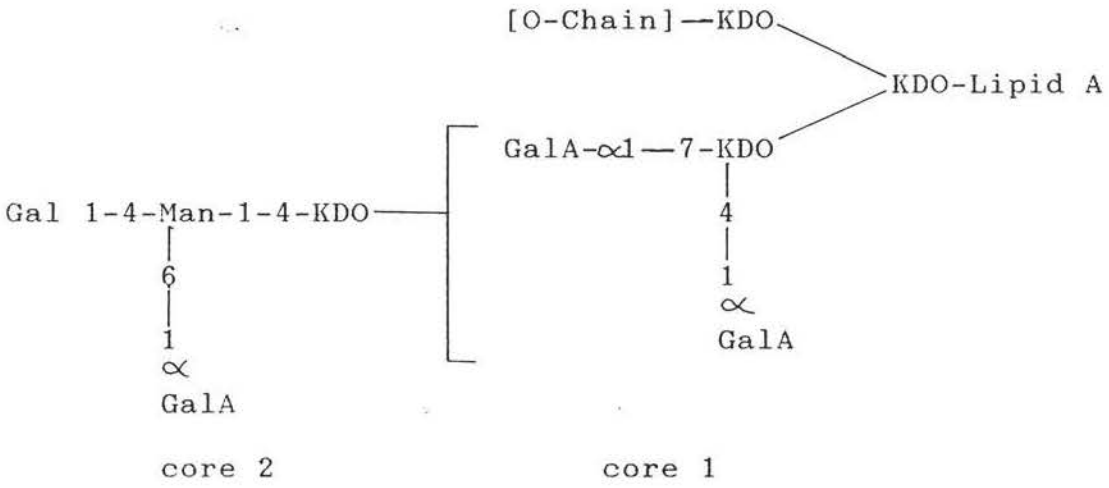

Figure 2. A proposed structural arrangement of the LPS components for R. leguminosarum biovar leguminosarum, biovar trifolii and biovar phaseoli.

reducing ends, indicating that both are linked to the remainder of the LPS molecule via a KDO residue (5).

Analysis of the LPS from specific symbiotic mutants should aid in determining what role, if any, the LPSs play in the symbiotic process. Several studies have reported changes in the composition of LPSs from bv. trifolii Nod- mutants which lack the symbiotic (Sym) plasmid or have a deletion in this plasmid (5,14,22,23). Noel et al have described two bv. phaseoli mutants that are defective in nodule development (Ndv-) and have altered LPS. These mutants form nodules that contain infection threads and bacteria; however, the infection threads exhibit abnormal morphology and abort. Both defects, in LPS and nodule development, appear to be due to a single mutation in each mutant. PAGE analysis of the mutant LPSs revealed that they lacked a higher-molecular-weight LPS I band, but contained a lower-molecular weight LPS II band which was present in both the parent and the mutant LPSs. LPS I is the complete form of the LPS that carries the O-antigen, while LPS II is the incomplete form of the LPS that lacks the O-antigen (4,5).

In this study, the isolation and partial characterization of the LPSs from R. leguminosarum biovar trifolii parent strain TB4 and two mutant strains, TB104 and TB112, are described. Bv. trifolii TB4 is a Nal^r Str^r derivative of bv. trifolii ANU843. Mutants TB104 and TB112 originated by random insertion of transposon Tn5 by means of suicide

plasmid pSUP2021 (36). Transposon Tn5 carries kanamycin resistance as a marker gene. Table 1 gives relevant characteristics of these bacterial strains.

Table 1

Bacterial Strains

Strain	Phenotype	Antibiotic Resistence
TB4	Hac^{+} [a] Nod^{+} [b] Fix^{+} [c]	Nal^{r} [d] Str^{r} [e]
TB104	Hac^{+} Nod^{+} Fix^{-}	Nal^{r} Str^{r} Kan^{r} [f]
TB112	Hac^{+} Nod^{+} Fix^{-}	Nal^{r} Str^{r} Kan^{r}

[a] Root Hair curling. [b] Nodulation-proficient. [c] Nitrogen fixation proficient. [d] Nalidixic acid resistant. [e] Streptomycin resistant. [f] Kanamycin resistant.

MATERIALS AND METHODS

Organisms

Bacterial strain *R. leguminosarum* biovar *trifolii* TB4 is a Nal^r Str^r derivative of *R. leguminosarum* biovar *trifolii* ANU843. Biovar *trifolii* TB4 and its mutants TB104 and TB112 were obtained from Dr. Dale Noel of Marquette University.

Growth conditions

The bacteria were grown in 10L batches at 25°C in Tryptone Yeast Medium (TY) and aerated with filter-sterilized air. This medium represses the production of extracellular polysaccharides (3). Table 2 lists the ingredients

Table 2

Tryptone Yeast Medium

Ingredient	Amount
Tryptone	0.5%
Yeast Extract	0.3%
$CaCl_2$	10mM

The pH was adjusted to 6.8.

of TY medium. Bacterial slants were checked for purity by gram stain (24) before inoculating four 125 mL starter flasks containing 40 mL of TY medium. The flasks were placed on a shaker for 4-5 days at 25°C. One day before

transfer to a 10L bottle of TY medium, each starter flask was tested by Gram staining, for growth on TY medium containing the appropriate antibiotics (Table 1), and for their inability to grow on nutrient agar. There was no evidence of contamination.

The contents of the starter flasks were then transferred to 10L of sterilized TY medium, incubated at 25°C, and aerated with filter-sterilized air. Two days prior to harvesting, the purity tests described above were performed. Bacterial growth was monitored by measuring the optical density at 620 nm. The bacteria were harvested at early stationary phase by centrifugation at 7000 rpm, using a Sorvall GSA rotor, for 20 minutes at 4°C. The pellets were collected and combined into a vial and stored at -20°C until used.

Due to low amounts of detectable hexose, uronic acid, and KDO in subsequent testing, samples of the two mutant strains were also obtained from Dale Noel of Marquette University. These two mutant strains were grown in 100L batches using a fermentor at the University of Wisconsin. Dried cells of TB104 were received which had a wet pellet weight of 425.41g. A 139mg water phase sample of TB112 was received. In addition, a 20L batch of TB112 was grown in my lab.

Isolation of Lipopolysaccharides (LPS)

The LPSs were isolated by the hot phenol-water extraction procedure (25,26) (Appendix I). The bacterial pellets were suspended in 100 mL of 65°C deionized water and extracted with 100 mL of 90% phenol at 65°C. This slurry was stirred for 15 minutes at 65°C. Then, the mixture was cooled in an ice bath for 15 minutes and centrifuged at 7,000 rpm for 20 minutes. The water layer containing the LPS was removed and saved. This procedure was repeated and the phenol layer was extracted again using a fresh supply of water. The water layers were then combined. When extracting the pellet from the 20L batch of TB112 and the 100L batch of TB104, 500 mL of hot phenol and 500 mL of hot water were used instead of 100 mL of each and the extraction procedure was performed three times instead of two times.

The combined water layers were dialyzed against deionized water in Spectra/Por membrane tubing with a molecular weight cutoff of 12,000-14,000 to remove phenol residues. Since it was unknown whether the LPSs from the two mutant strains would be soluble in the water layer or the phenol layer, the phenol layers from TB104 and TB112 were further purified. The phenol layers, including the precipitate, were dialyzed against deionized water in Spectra/Por membrane tubing with a molecular weight cutoff of 12,000-14,000. The water was changed many times in order to remove the phenol. The pellet suspensions were then lyophilized,

with the weight of TB104 and TB112 being 5.10g and 3.81g, respectively. Next, 50 mL of deionized water were added to each pellet and the mixture was blended in a Waring blender for five minutes. Then, proteinase K was added in a proportion of 10 mg/100mL and the mixture was placed on a shaker for 24 hours. The mixture was then dialyzed as before, and centrifuged. The pellet was frozen and the supernatant was concentrated on a Buchi Rotavapor evaporator to about 50 mL and then lyophilized.

Purification of the Lipopolysaccharides

In addition to LPS, the water layers also contained DNA, RNA, and other molecules of low molecular weight such as glucan. The DNA and RNA were removed using the enzymes DNase I and RNase A. These enzymes hydrolyze the DNA and RNA into nucleic acids which are small enough to be removed by subsequent dialysis. The nuclease treatment was performed as shown in Table 3. The mixture was stirred and

Table 3

The Nuclease Treatment

Component	Concentration	mL/100mL LPS layer
DNase	1mg/mL in buffer	1
RNase	10mg/mL in buffer	1
buffer	0.1M Tris and 0.01M $MgSO_4$, pH=7.2	10

allowed to stand at room temperature for 24 hours. Then,

the mixture was dialyzed as before, concentrated, and lyophilized.

The resultant material was subjected to gel-filtration chromatography for complete purification of the LPS (25). The LPS sample was dissolved in the eluting solvent and applied to a 1.5 cm x 150 cm column of Sepharose 4B gel. The eluting solvent was a buffer at pH 7.0 containing 2.922 g/L of ethylenediamine tetraacetic acid (EDTA), 4.3 mL/L of triethylamine (TEA), and 200 mg/L of sodium azide. The flow rate was about 8 mL/hr. Since the LPS contains hexose and KDO, the peaks were detected by performing the hexose (24) and KDO (27) assays (Appendices II and III). The peak containing both hexose and KDO corresponded to pure LPS (4). The fractions containing the LPS were collected, dialyzed to remove the buffer, and lyophilized. The resulting lyophilized material was crystalline due to the triethylamine buffer still present. So, the LPS material was dissolved in 50 ml of deionized water. Cation exchange resin, Dowex 50 H^+ form, was added, mixed for 1 hour, filtered out, and the LPS was re-lyophilized. The resulting LPS samples were then fluffy white.

Composition Analysis

The hexose compositions were determined by gas chromatography (GC) of the alditol acetate derivatives on a Hewlett-Packard GC (Model 5890) equipped with a 0.2 um df by

15m Fused Silica Capillary SP-2330 column. The polysaccharides were acid hydrolyzed by heating with 2M trifluoroacetic acid (TFA). The resulting monosaccharides were then reduced to alditols with sodium borohydride and then converted to alditol acetates by heating with acetic anhydride in pyridine (Appendix IV). Identification and quantitation was done by comparison of the GC retention times and the peak areas to standard sugars. The amount of each sugar was determined by integrating the curve generated by the flame ionization of the gas chromatographic effluent. The integration was accomplished with a computer data system, Chromatochart, by IMI, State College, PA. Inositol was used in all samples as an internal standard (14,28) (Appendix IV).

Uronic acids were quantitated by the colorimetric assay of Blumenkrantz and Asboe-Hansen, using glucuronic acid as the standard (29) (Appendix VI). The uronic acids were identified by reducing the carboxyl groups prior to acetylation analysis. The polysaccharide was treated with HCl in methanol, reduced with $NaBH_4$, hydrolyzed, and acetylated as described above (Appendix V). Increases in, or the appearance of, a particular hexose over that of the noncarboxyl reduced sample showed that the hexose was a hexuronic acid in the noncarboxyl reduced polysaccharide (14). KDO, acetyl groups, phosphate, and pyruvate were also assayed by colorimetric assays (27,30,31) using KDO, glucose penta-

acetate, sodium phosphate (dibasic), and pyruvate as standards, respectively (Appendices III, VII, VIII, and IX).

Polyacrylamide Gel Electrophoresis

The LPS samples were analysed by polyacrylamide gel electrophoresis (PAGE) as described in Appendix X. A 1.0 mg/mL solution of each LPS sample was prepared. A sample of Salmonella LPS was also included for calibration of the gel. 40 uL of each solution were lyophilized, and then redissolved in 25uL of sample buffer. These solutions were then heated in sealed tubes at 100°C for 5 minutes. After cooling to room temperature, the samples were run on a 15% polyacrylamide gel containing deoxycholic acid (DOC) (32) as a detergent. The current was kept constant at 18 ma through the stacking gel and 25 ma through the running gel. The gel was run on a 16 cm Hoefer Scientific Instruments Sturdier SE 400 Slab Unit. Running time was around 4 hours. When the bromphenol blue dye reached the bottom of the gel, the gel was removed from the apparatus and stained for LPS by the silver staining method of Tsai and Frasch (33) (Appendix XI). The stained gel was stored in plastic wrap at 4°C.

Immunoblot Procedure

The LPSs from a slab gel, which was run as described above, were electroblotted onto nitrocellulose paper using a Hoeffer Transphor unit. The voltage was set at 80V for 1 hour. The transfer buffer was 25mM tris/187mM glycine

prepared in 20% methanol solution. The nitrocellulose paper was removed, rinsed in deionized water, and allowed to dry. The nitrocellulose paper was then stained for bv. trifolli O-antigen using ANU843 antisera and peroxidase conjugated antirabbit goat antisera (Sigma Chemical Co.). The paper was developed with peroxide and 4-chloro-1-naphtol (14) (Appendix XII).

RESULTS

Lipopolysaccharide Purification

The isolation of LPS from R. leguminosarum bv.trifolii TB4 and mutants TB104 and TB112 using the hot phenol-water extraction gives a low yield, particularly with the two mutant strains, as seen in Table 4. Furthermore, purification by RNase and DNase along with gel filtration on a Sepharose 4B column lowers the yield even more.

Table 4

LPS Yields of bv. trifolii strains

Strain	wet weight of bacterial pellet g/10L culture	water layer extracted LPS g/10L culture	Pure LPS g/10L culture
TB4	20.0	0.196	0.147
TB104	45.4	0.0378	0.0176
TB112	27.4	0.0371	0.0184

The LPS extracted with the phenol-water extraction procedure is contaminated with glucose-rich polysaccharides and DNA and RNA fragments. For this reason, the LPS sample is purified by gel filtration on Sepharose 4B by eluting with an EDTA- and TEA-containing buffer (25). This buffer chelates the cations, usually Ca^{2+} and Mg^{2+}, which maintain large aggregation states of LPS (16,34,35). The Sepharose 4B separates the higher molecular weight LPS from the lower molecular weight contaminants. The eluted 8ml fractions are assayed for hexose and KDO. In assaying the water layer fractions of TB4, TB104, and TB112, the first peak from the

4B column, nearest the void volume, contains both hexose and KDO and corresponds to pure LPS (25) (see Figures 3, 4a, and 5a). These fractions are collected, dialyzed as described before, and lyophilized. In assaying the phenol layer fractions of TB104 and TB112, negligible amounts of KDO are detected, as seen in Figures 4b and 5b. Therefore, the LPS in the two mutant strains appears to be soluble in the water layer and not the phenol layer.

Polyacrylamide Gel Electrophoresis of Lipopolysaccharides

The LPS samples of bv. trifolii TB4, TB104, and TB112 are analyzed by polyacrylamide gel electrophoresis (PAGE) as described in the materials and methods section of this paper and in Appendices X and XI. A reproduction of one gel is found in Figure 6. The Salmonella LPS control in well 1 shows the characteristic ladder-like banding in the large molecular weight region a. This banding pattern has been suggested to be due to LPS molecules differing from one another by one O-antigen repeating unit (14).

Biovar trifolii TB4 in well 2 shows a broad band and two narrow bands in region a. The multiple bands are probably due to differing aggregation states of complete LPS, LPS I, which may contain a relatively short O-antigen polysaccharide rather than a long repeating oligosaccharide (14). Bv. trifolii TB104 and TB112, in wells 3 and 4 respectively, are completely missing the LPS I bands. The

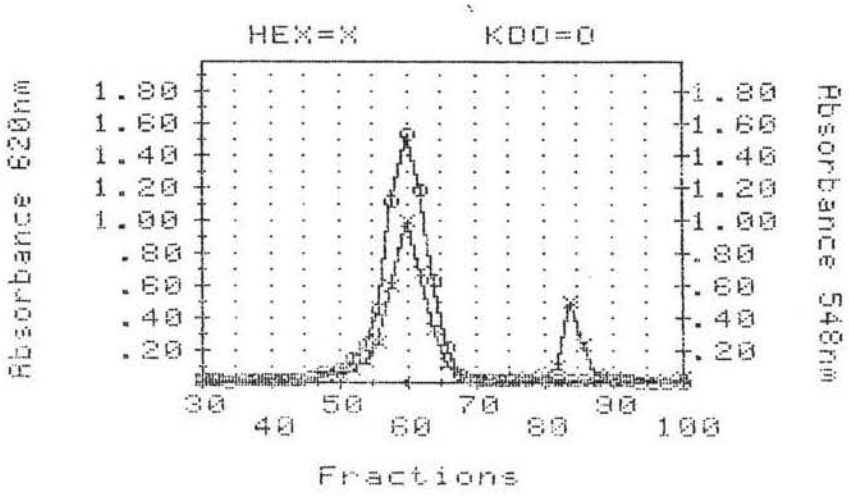

Figure 3. Sepharose 4B EDTA-TEA gel filtration column of bv. trifolli TB4 LPS. Fractions (8ml) were assayed for hexose by the anthrone assay, and for KDO by the thiobarbituric assay.

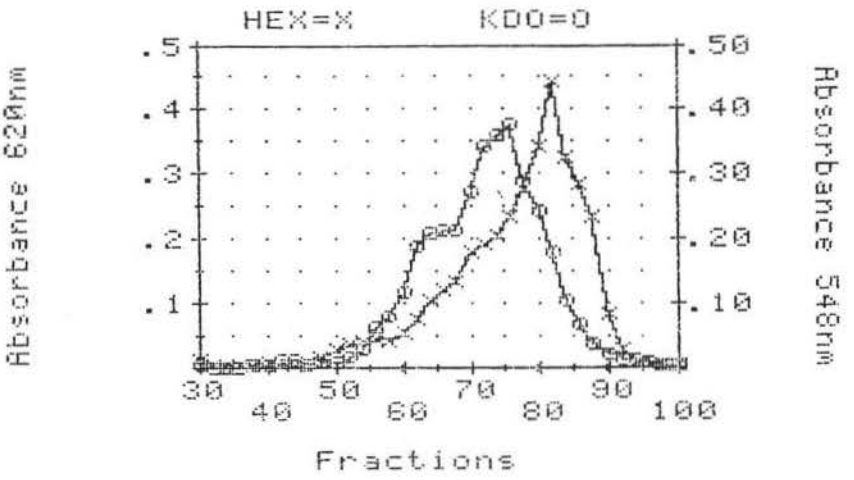

Figure 4a. Sepharose 4B EDTA-TEA gel filtration column of bv. trifolii TB104 LPS, water layer. Fractions (8ml) were assayed for hexose by the anthrone assay, and for KDO by the thiobarbituric assay.

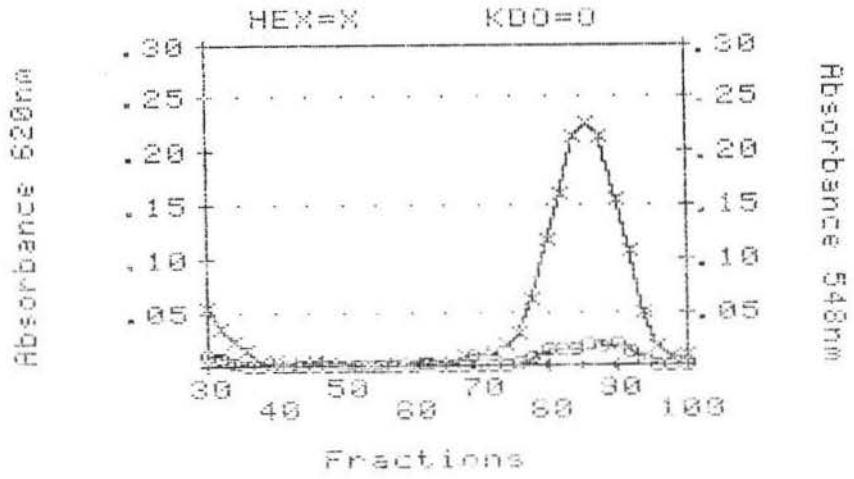

Figure 4b. Sepharose 4B EDTA-TEA gel filtration column of bv. trifolii TB104 LPS, phenol layer. Fractions (8ml) were assayed for hexose by the anthrone assay, and for KDO by the thiobarbituric assay.

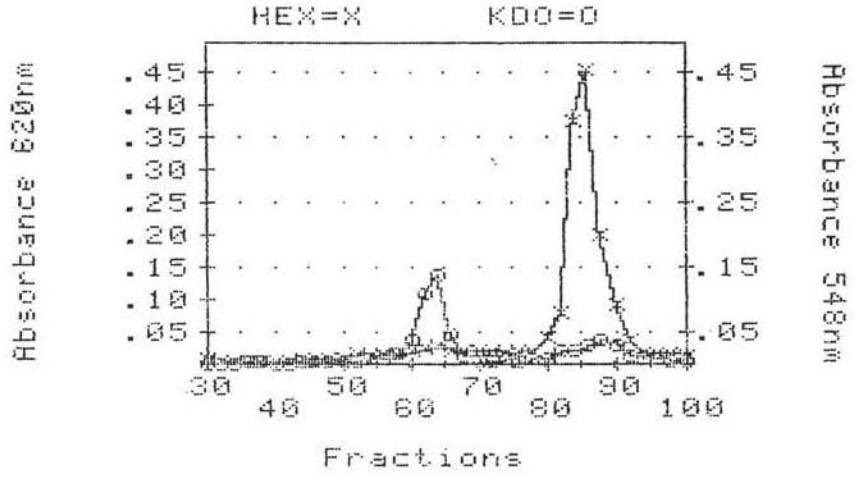

Figure 5a. Sepharose 4B EDTA-TEA gel filtration column of bv. trifolii TB112 LPS, water layer. Fractions (8ml) were assayed for hexose by the anthrone assay, and for KDO by the thiobarbituric assay.

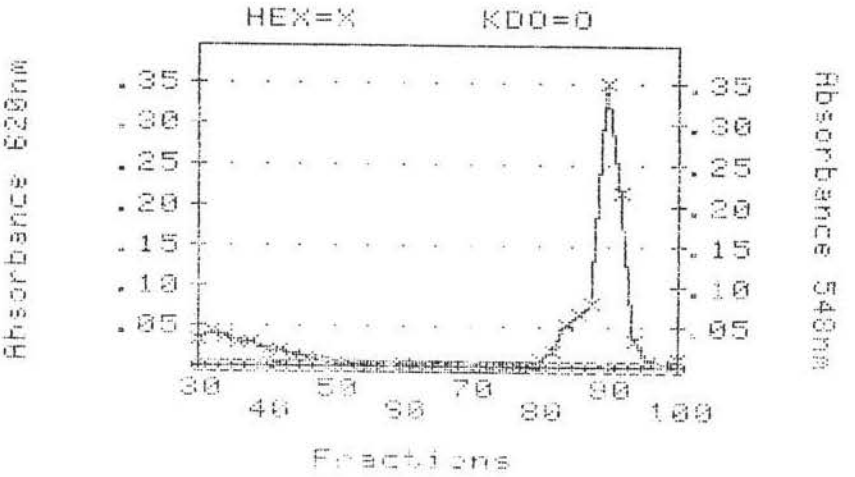

Figure 5b. Sepharose 4B EDTA-TEA gel filtration column of bv. trifolii TB112 LPS, phenol layer. Fractions (8ml) were assayed for hexose by the anthrone assay, and for KDO by the thiobarbituric assay.

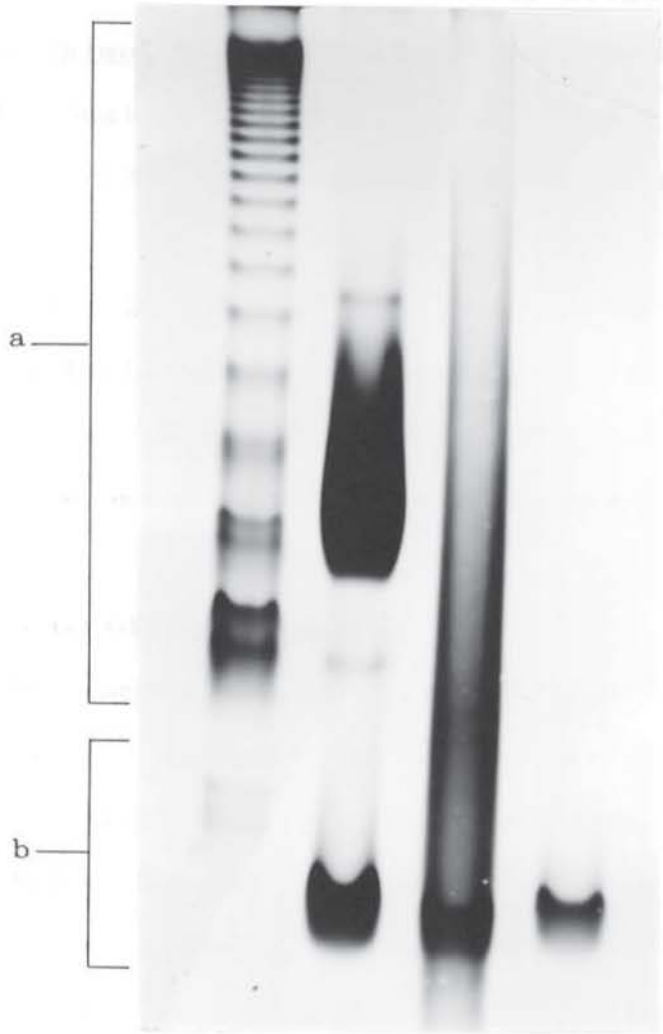

Figure 6. 16 cm, 15% acrylamide DOC-PAGE gel. Wells: 1) Standard, Salmonella LPS; 2) bv. trifolii TB4 LPS; 3) bv. trifolii TB104 LPS; 4) bv. trifolii TB112 LPS. Letters refer to band regions (see text).

missing O-antigen in TB104 and TB112 is confirmed by the immunoblot prodecure (immunoblot not shown) as described in the materials and methods section of this paper and in detail in Appendix XII. The bv. trifolii ANU843 antisera reacts only with the immunodominant O-antigen broad band in region a of TB4 and does not react with TB104 or TB112.

Region b consists of single, somewhat broad lower molecular weight bands with mobility slightly greater than that of the Salmonella control. It has been suggested that this band consists of the lipid A-core oligosaccharide portion of the LPS, i.e., the incomplete form of LPS, LPS II (14).

PAGE analysis of phenolic layer Sepharose 4B fractions of TB104 and TB112 shows no bands, (PAGE gel not shown) confirming that the LPS from these two mutant strains is soluble in the water layer and not the phenol layer.

Lipopolysaccharide Compositions

The LPS compositions are given in Table 5. The bv. trifolii parent strain, TB4, LPS contains the O-antigen sugars 2-O-methyl-6-deoxyhexose, fucose, 3-N-methyl-3-amino-3,6-dideoxyhexose, heptose, and 2-amino-2,6-dideoxy-hexose. The bv. trifolii mutant strains TB104 and TB112 are missing all these sugars. This result corresponds well with the PAGE and immunoblot results given earlier. TB4 has a large amount of glucuronic acid and galacturonic acid; TB104

Table 5

LPS Compositions of bv. *trifolii* parent and mutants

Components	TB4	TB104	TB112
2-O-methyl-6-deoxyhexose	3.5±0.2 (2.7±0.3)	0 (0)	0 (0)
fucose	1.5±.07 (1.3±.07)	0 (0)	0 (0)
xylose	0 TR	0 (0.6±0.1)	TR (0.6±.07)
mannose	2.5 (2.7±.07)	3.0±0.2 (2.3±0.7)	2.6±0.1 (1.3±.07)
galactose	2.3±0.1 (3.5±0.3)	TR (0.7±0.1)	TR (0.7±.07)
glucose	0 (2.3±0.6)	TR TR	1.3±0.1 (0.9)
3-N-methyl-3-amino-3,6-dideoxyhexose	5.5±.07 (2.8±0.7)	0 (0)	0 (0)
heptose	7.7±0.1 (6.9±3.1)	0 (0)	0 (0)
2-amino-2,6-dideoxyhexose	2.8±0.2 (2.1)	0 (0)	0 (0)
2-keto-3-deoxyoctonate (KDO)	7.5±0.3 (ND)	1.5±0.2 (ND)	2.6±0.3 (ND)
uronic acid	10.8±3.4 (ND)	TR (ND)	TR (ND)
acetyl groups	0.9±.04 (ND)	5.1±1.6 (ND)	1.3±0.8 (ND)
phosphate groups	0 (ND)	8.8±0.9 (ND)	0 (ND)
pyruvyl groups	0 (ND)	0 (ND)	0 (ND)

The compositions are given in percent of mass. The components account for 45, 19, and 9% of the LPS mass from strains TB4, TB104, and TB112, respectively. The standard deviations are calculated from two analyses of the same

sample for all the components except for KDO, uronic acid, phosphate groups, and pyruvyl groups, which are calculated from six analyses of the same sample. The values in parentheses are of the uronic acid reduced LPSs. ND, not determined; TR, trace. The detector response factors for fucose, galactose, and heptose were used to estimate the amounts of 2-O-methyl-6-deoxhexose, 3-N-methyl-3-amino-3,6-dideoxyhexose, and 2-amino-2,6-dideoxyhexose, respectively.

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and TB112 have only small amounts, if any, of these uronic acids present. The major sugar in both mutant strains is mannose. Figures 7, 8, and 9 show the gas chromatograms of TB4, TB104, and TB112, respectively. Parent strain TB4 has a much larger amount of KDO than the two mutant strains. Conversely, mutant TB104 has a much larger amount of acetyl groups than TB4 or TB112, and TB104 has a large amount of phosphate whereas TB4 and TB112 had no detectable amount. In addition, neither TB4 nor the mutant strains have any detectable amount of pyruvyl groups.

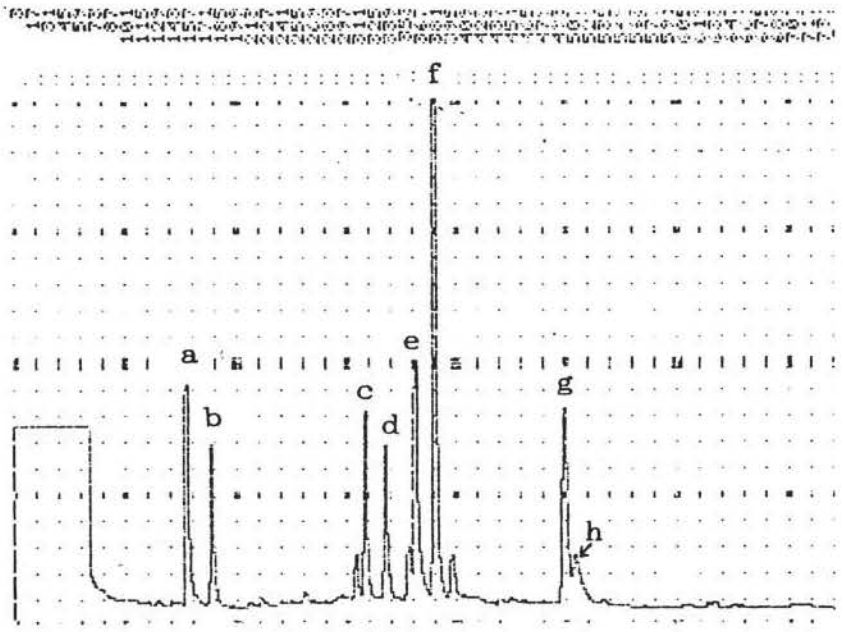

Figure 7a. Gas chromatograms of bv. trifolii TB4 hexose alditol acetate derivatives. a, 2-O-methyl-6-deoxyhexose; b, fucose; c, mannose; d, galactose; e, 3-N-methyl-3-amino-3,6-dideoxyhexose; f, inositol; g, heptose; h, 2-amino-2,6-dideoxyhexose.

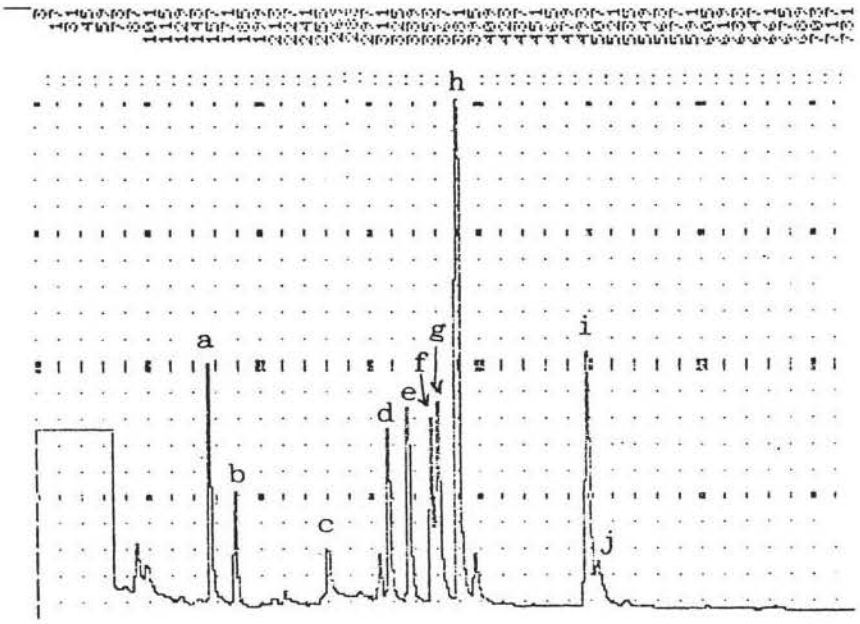

Figure 7b. Gas chromatograms of carboxyl reduced bv. trifolii TB4 hexose alditol acetate derivatives. a, 2-O-methyl-6-deoxyhexose; b, fucose; c, xylose, d, mannose; e, galactose; f, glucose; g, 3-N-methyl-3-amino-3,6-dideoxyhexose; h, inositol; i, heptose; j, 2-amino-2,6-dideoxyhexose.

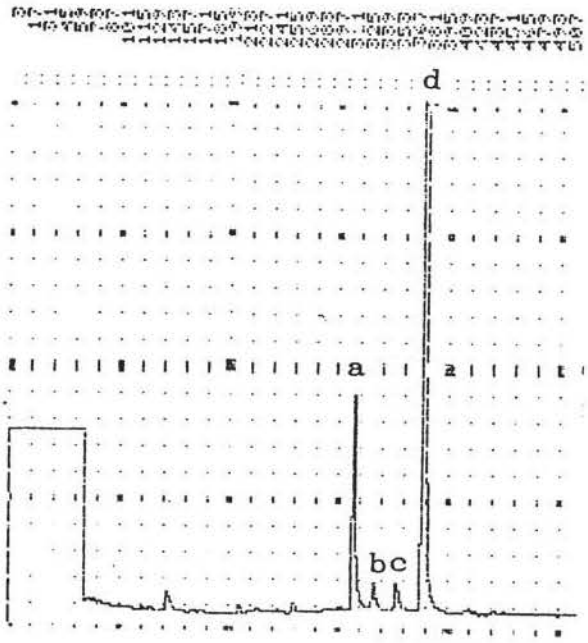

Figure 8a. Gas chromatograms of bv. trifolii TB104 hexose alditol acetate derivatives. a, mannose; b, galactose; c, glucose; d, inositol.

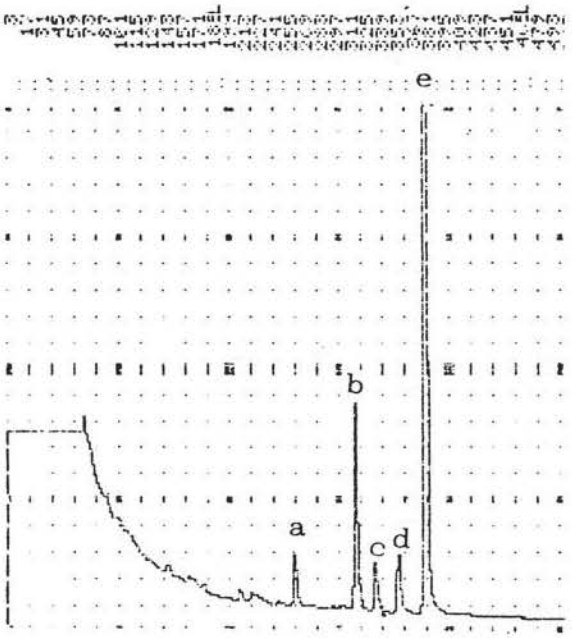

Figure 8b. Gas chromatograms of carboxyl reduced bv. trifolii TB104 hexose alditol acetate derivatives. a, xylose; b, mannose;, c, galactose; d, glucose; e, inositol.

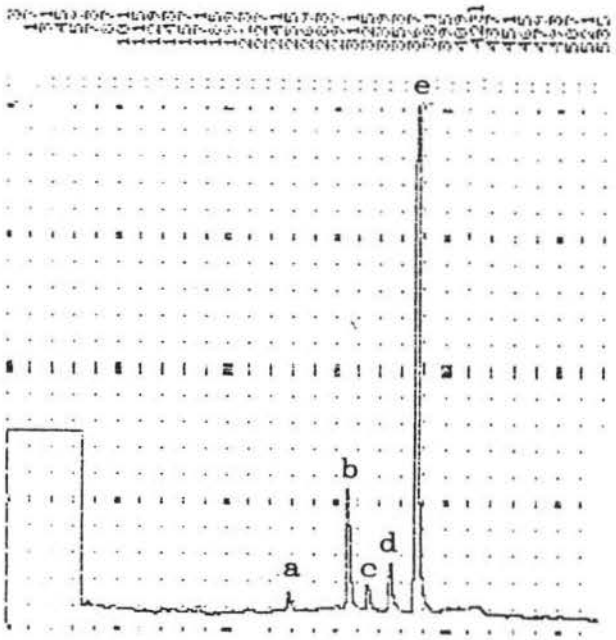

Figure 9a. Gas chromatograms of bv. trifolii TB112 hexose alditol acetate derivatives. a, xylose; b, mannose; c, galactose; d, glucose; e, inositol.

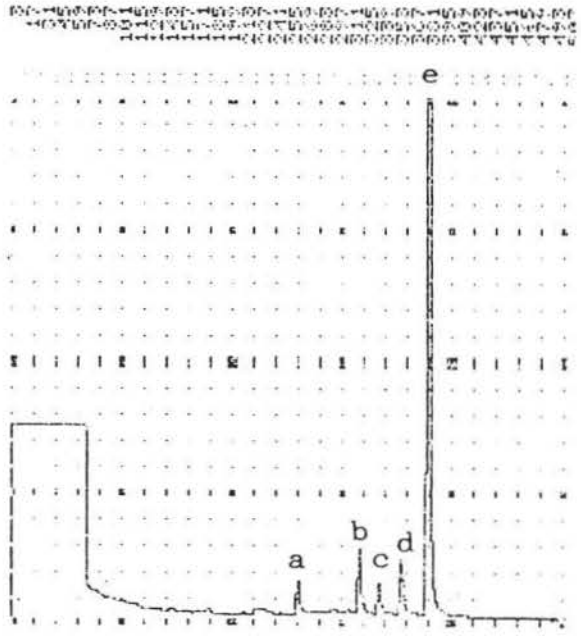

Figure 9b. Gas chromatograms of carboxyl reduced bv. trifolii TB112 hexose alditol acetate derivatives. a, xylose; b, mannose; c, galactose; d, glucose; e, inositol.

DISCUSSION

The results of this study confirm that the two mutant strains R. leguminosarum bv. trifolii TB104 and TB112 are missing LPS I, the complete form of LPS containing the O-antigen chain. Chemical analysis of the LPS of these two mutants shows that they lack the O-antigen sugars, i.e. 2-O-methyl-6-deoxyhexose, fucose, 3-N-methyl-3-amino-3,6-dideoxyhexose, heptose, and 2-amino-2,6-dideoxyhexose, which are found in the parent strain TB4. In addition, PAGE analysis verifies that the mutants are missing the O-antigen through the lack of bands in the LPS I higher molecular weight region. Furthermore, the immunoblot procedure revealed that the bv. trifolii ANU843 antisera reacted only with the parent strain TB4, which has the complete form of LPS. Thus, while the parent strain, TB4, contains both LPS I and LPS II, the mutant strains, TB104 and TB112, contain only LPS II.

Phenotypically, both TB104 and TB112 are $Nod^{+}Fix^{-}$. More specifically, the mutants are Ndv^{-}, i.e., they lack nodule development. These mutants initiate infection of the legume, but then abort the infection threads. They elicit incompletely developed clover nodules that exhibit low bacterial populations and very low nitrogenase activity (36, Noel, personal communication). Bv. trifolii TB104 and TB112 are similar to R. leguminosarum bv. phaseoli mutants which

have been previously reported. Like TB104 and TB112, the bv. phaseoli mutants were formed from Tn5 transposon mutagenesis of a parent wild type strain. These mutants have altered LPS, missing the O-antigen, and they abort their infection threads. Both defects appear to be due to the same mutation (4,5,20). Possibly the mutation affects nodule development because LPS I content or structure is altered (20).

It appears that the ability to synthesize LPS molecules containing the O-antigen is essential for carrying infection beyond an early stage. However, normal LPS does not appear to be required for earlier events in nodulation, such as attachment to the root surface, root hair curling, and induction of root cortical-cell division to produce a nodule structure (5).

The role of LPS I has yet to be determined. One possibility is that LPS I protects against host defenses that confront the bacteria only after the infection thread is initiated. LPS I may mask features which elicit host defenses that stop the infection process. Another possibility is that LPS I prevents entry of host toxins. Furthermore, the O-antigen, or a nodule-specific modification of it, may serve as a signal molecule that is recognized by the plant, which triggers a step in infection thread synthesis and thus sustains infection (5,20).

The molecular effects of the mutation on the LPS

structures of bv. trifolii TB104 and TB112 remain unknown. Besides missing the O-antigen, the core structures of the LPS could be affected. Mild-acid hydrolysis of bv. trifolii ANU843 releases polysaccharide fractions, PS1, PS2, and PS3. PS1 is the immunodominant O-antigen. PS2 is the core 2 and PS3 is the core 1 (14,5,Carlson personal communication). (Refer to Figure 2 in the introduction section of this paper for the proposed molecular structure of these polysaccharide fractions.) Research with bv. phaseoli CE109 and CE309, both mutants of bv. phaseoli CE3, shows that CE109 is defective in both core 1 (PS3) and core 2 (PS2), while CE309 has normal core 1 but is defective in core 2. Since both bv. trifolii TB104 and TB112 are very low in uronic acid, i.e., only a trace amount was detected in each, it is highly likely that the galacturonic acid-rich core is altered in its structure.

In addition, cosmid pCOS109.11, which corrects the CE109 mutant, also corrects TB112 to a bv. phaseoli type of LPS. Phenotypically, cosmid pCOS109.11 also corrects Nod$^+$Fix$^-$ to Nod$^+$Fix$^+$ in clover. However, cosmid pCOS109.11 does not correct TB104. Conversely, another bv. phaseoli cosmid, pCOS126, corrects TB104 to a bv. trifolii type of LPS. Phenotypically, cosmid pCOS126 corrects Nod$^+$Fix$^-$ to Nod+Fix$^+$ in clover. Cosmid pCOS126 does not correct TB112. These results suggest that the core regions altered in TB112 are different than those altered in TB104. It is known that

pCOS109.11 carries genes for part of the core region plus the bv. phaseoli O-antigen. Apparently, pCOS126 carries genes for the core region only. Genes for the O-antigen in TB104 are intact and do not require correction (20,36, Carlson personal communication).

Additional research needs to be done to further characterize the LPS structures of R. leguminosarum bv. trifolii TB4, TB104, and TB112. Mild-acid hydrolysis of the LPSs would allow further study of the resulting polysaccharide fractions (PS1, PS2, and PS3). These fractions could then be characterized by acetylation and methylation analysis and by nuclear magnetic resonance spectroscopy.

Appendix I

LPS extraction (Hot phenol/water method)

Reagents:

A. 90% phenol, liquid

B. Deionized water

C. DNase I (1mg/mL in 0.1M Tris and 0.01M $MgSO_4$ at pH 7.2)

D. RNase A (10mg/mL in 0.1M Tris and 0.01M MgSO4 at pH 7.2)

E. 0.1M Tris and 0.01M $MgSO_4$ at pH 7.2

F. Proteinase K

Procedure:

1. In hood, set up a water bath at 65°C.
2. Warm 100mL (500mL) phenol in one container and enough dH_2O in another which when added to the bacteria will equal approximately 100mL (500mL). Use 500 mL if sample volume is large.
3. Add warm water to bacteria by washing with small portions so that all the bacteria are recovered. Add warm phenol to this and warm with stirring to 65°C and heat there for 15 mins. Cool in an ice bath for 15 mins. Centrifuge for 20 mins. and separate the top water layer by using a Pasteur pipette. Save the water layer. Repeat the procedure by reheating the phenol, using a fresh supply of water, and adding the bacteria to this water. (Repeat again if using 500mL).
4. Combine the water layers and continue with steps 5-9.

Save the phenol layer and continue with steps 10-14.

5. Dialyze the water layer against dH_2O changing the water at least 4 times at approximately 6 hour intervals.
6. Centrifuge to remove waste.
7. Add 0.1mL per 100mL of DNase and RNase and 10mL per 100ml of reagent E. Mix. Allow to stand at room temperature 24 hours.
8. Dialyze against dH_2O. Change water at least 4 times at approximately 6 hour intervals.
9. If necessary, centrifuge to remove waste and concentrate. Freeze dry. Weigh and store in freezer.
10. Dialyze the phenol layer against dH_2O changing the water many times at approximately 6 hour intervals until phenol odor is gone.
11. Concentrate supernatant. Blend supernatant and remaining bacterial pellet in Waring blender for 5 min.
12. Add proteinase K (10mg/100mL) and shake for 24 hours.
13. Dialyze against dH_2O. Change water at least 4 times at approximately 6 hour intervals.
14. Centrifuge to remove waste and concentrate. Freeze dry. Weigh and store in freezer.

Appendix II

Hexose Assay

Reagents:

A. 0.2% anthrone in conc. H_2SO_4

B. Standard: 1 mg/mL solution of glucose

C. Samples: 1 mg/mL solution

Procedure:

1. Prepare a set of standards from 0 to 200 uL and q.s. to 500 uL with deionized water.
2. Prepare a series of tubes for the samples but use larger volumes, 50-300 uL, and q.s. to 500 uL with deionized water.
3. Add 1 mL of reagent A. Vortex and wait 5 min.
4. Read absorbance at 620 nm.

Appendix III

KDO (2-keto-3-deoxyoctonic acid) Assay

Reagents:

A. 10M H_2SO_4

B. 0.04N HIO_4 in 0.4N H2SO4

C. 2% $NsAsO_2$ (sodium arsenite) in 0.5N HCl

D. 0.3% thiobarbituric acid

E. Standard: 0.1 mg/mL KDO

Procedure:

1. Prepare a set of standards from 0 to 100 uL and q.s. to 200 uL with deionized water.
2. Prepare a set of samples using 200 uL.
3. Add 20 uL of reagent A to standards and samples, vortex.
4. Place in boiling water bath for 1 hour.
5. Add 250 uL of reagent B. Vortex. Let stand at room temperature at least 40 mins.
6. Add 500 uL of reagent C. Vortex. Allow to stand 5 mins.
7. Add 2 mL of reagent D. Vortex. Place in a boiling water bath for 20 min.
8. Read absorbance immediately at 548 nm. Centrifuge any cloudy samples 1 min in a tabletop centrifuge.

Appendix IV

Acetylation Procedure

Reagents:

A. Inositol (1 mg/mL solution), as internal standard

B. Standard sugars (1 mg/mL solution): rhamnose, fucose, ribose, xylose, mannose, galactose, glucose, and heptose

C. 2.0M trifluoroacetic acid (TFA)

D. Sodium borohydride (or sodium borodeuteride): 10 mg/mL solution in 1M ammonium hydroxide

E. Glacial acetic acid

F. 10% glacial acetic acid in methanol

G. Methanol

H. Pyridine

I. Acetic anhydride

J. Chloroform

Procedure:

1. Determine the percent hexose in the sample using the anthrone assay.

2. Place sample equivalent to not more than 250 ug hexose in a screw-cap test tube. In another tube, place 100 uL of each standard. Add 20 uL of inositol to each tube. Dry materials by blowing filtered air or by freeze-drying.

3. Add 500 uL of TFA to each tube, seal with a teflon-lined screw cap, and heat at 121°C for 2 hours.

4. Remove tubes and blow dry with filtered air. This may be done in a water bath at 40-50°C. For methylated polysaccharides, blow dry at no more than 35°C.
5. Add 250 uL of sodium borohydride solution, mix and allow to stand at room temperature for 1 hr (can be left overnight with loose caps on). For methylated samples always use sodium borodeuteride, and leave at room temperature for 2 hrs.
6. Add 50 uL of glacial acetic acid. Vigorous bubbling will take place. Repeat two more times.
7. Add 500 uL of the 10% glacial acetic acid in methanol and blow dry with filtered air. Keep drying temperatures as mentioned previously. Do this four times.
8. Add 500 uL of methanol and blow dry as in step 7. Do this four times.
9. Add 50 uL of pyridine and 50 uL of acetic anhydride. Mix, seal tubes with teflon caps and heat at 121°C for 30 min.
10. Cool on ice, blow dry at room temperature, and extract by adding 500 uL of water and 500 uL of chloroform. Mix, centrifuge for 5 min in a table top centrifuge, remove chloroform layer (bottom) and transfer to another test tube.
11. Extract water layer with another 500 uL of chloroform and combine chloroform layers. Blow dry with filtered air. Analyze by gas chromatography.

Appendix V

Carboxyl Reduction Procedure

Reagents:

A. 0.5% HCl in methanol

B. 10mg/mL solution of sodium borohydride ($NaBH_4$) in ethanol

C. Glacial acetic acid

D. 10% glacial acetic acid in methanol

E. Methanol

Procedure:

1. Determine the percent hexose in the sample using the anthrone assay.
2. Place sample equivalent to not more than 250 ug hexose in a screw-cap test tube. Dry by blowing filtered air or by freeze-drying.
3. Add 500 uL of reagent A to each tube, seal with a teflon-lined screw cap, and heat at 50°C for 5 hours. The tubes may be left overnight at room temp, after the 5 hours, if necessary.
4. Blow dry with filtered air at 40°C.
5. Add 0.5 mL of reagent B. Allow to stand overnight at room temperature.
6. Heat at 70°C for 1 hour.
7. Add 50 uL of glacial acetic acid. Vigorous bubbling will take place. Repeat two more times.
8. Add 500 uL of the 10% glacial acetic acid in methanol

and blow dry with filtered air. Keep drying tempera ture at 40°C. Do this four times.

9. Add 500 uL of methanol and blow dry as in step 8. Do this four times.

10. Continue with the acetylation procedure as described in Appendix IV by adding the inositol in step 2.

Appendix VI

Uronic Acid Assay

Reagents:

A. 0.0125M Sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in conc. H_2SO_4. Stored at 4°C

B. 0.15% m-hydroxybiphenyl in 0.5% NaOH. Stored at 4°C

C. 0.5% NaOH

D. Standard glucuronic or galacturonic acid (0.1 mg/mL)

Procedure:

1. Prepare a set of standards 0-200 uL and q.s. to 200 uL with deionized water.
2. Prepare two sets of samples with a total volume of 200 uL.
3. Add 1.2 mL reagent A. Vortex.
4. Heat for 5 min. in a boiling water bath.
5. Cool 1-2 min. in cold water.
6. To the standards and one set of samples add 20 uL of reagent B. To the other set of samples add 20 uL of reagent C. Vortex.
7. Allow to stand at room temperature for 5 min.
8. Read absorbance at 520 nm. Subtract any reading of the samples treated with NaOH from those treated with m-hydroxybiphenyl for the true reading.

Appendix VII

Assay for Acetyl Groups

Reagents:

A. 2M Hydroxyaminehydrochloride. Stored at 4°C

B. 3.5M NaOH

C. HCl, 1 part conc. HCl + 2 parts H_2O

D. 0.37M $FeCl_3 \cdot 6H_2O$ in 0.1M HCl

E. CH_3OH

F. Standards: 0.5 mg/mL B-D(+)-glucose pentacetate in CH_3OH. Stored at 4°C. 10 uL=2.75 ug acetate.

Procedure:

1. Mix equal parts of reagents A and B just before using. This is stable for three hours at room temperature.
2. Prepare series of standard and samples to a total volume of 400 uL.
   a. Standards: __uL (recommended 0, 10, 20, 40, 80, 100 uL) + CH_3OH to 100 uL + 300 uL dH_2O.
   b. Samples usually contain small quantities therefore use 200 or 300 uL or freeze dry 400 to 600 uL quantities. Add 100 uL CH_3OH to each and enough dH_2O for a final volume of 400 uL.
3. Add 800 uL of reagent prepared in step 1. Vortex and allow to stand for 1 minute.
4. Add 400 uL of reagent C (HCl). Vortex.
5. Add 400 uL of reagent D ($FeCl_3$). Vortex.
6. Measure absorbance at 540 nm.

Note: If samples contain significant quantities of protein, it will precipitate out upon addition of reagent D and may be removed by centrifuging.

Appendix VIII

Phosphate Assay

Reagents:

A. Standard: 0.01M Na_2HPO_4 diluted 1:20 in H_2O.

B. 10% $Mg(NO_3)_2$ in 95% ethanol.

C. 0.5M HCl

D. 10% ascorbic acid, freshly made.

E. 0.42% $NH_4Mo \cdot 4H_2O$ in 1M H_2SO_4. Mix 1 part of reagent D to 6 parts of reagent E. Store on ice.

Procedure:

1. Add 30 uL of reagent B to 150 uL of samples (50-150 uL of 1 mg/mL, q.s. to 150 uL with dH_2O) and standards (0, 5, 10, 20, 40, 60, 80, 100, and 150 uL, q.s. to 150 uL with dH_2O).
2. Dry samples and standards in an oven (ca. 80°C).
3. Remove from oven and shake over a flame for 5-10 seconds. Cool to room temperature.
4. Add 30 uL of reagent C and heat in a boiling water bath for 15 min.
5. Add 700 uL of 1:7 dilution of reagent D and reagent E.
6. Heat at 45°C in a water bath for 20 min.
7. Add 1 mL of H_2O and read absorbance at 820 nm.

Appendix IX

Pyruvic Acid Assay

Reagents:

Pyruvic acid standard and DNP must be made fresh the day they are to be used.

A. Standard: 0.1 mg pyruvic acid/1.0 mL H_2O

B. 2.0 M HCl

C. 500 u moles (99mg) of 2,4-dinitrophenylhydrazine (DNP) in 100 mL of 2.0M HCl. Dissolve at 40°C.

D. Toluene

E. 10% Na_2CO_3

F. H_2O

G. 2.2M NaOH

Procedure:

1. Set heating block at 100°C.
2. Prepare a set of standards 0-200 uL with a total volume of 200 uL. Use small screw top test tubes.
3. Prepare samples with a total volume of 200 uL. Usually less than 100 uL is not detectible. If samples contain protein treat with perchloric acid.
4. Add 300 uL of reagent B (HCl), vortex, and heat in sealed tubes in 100°C heating block for 3 hours.
5. Add 100 uL of reagent C (DNP), vortex, and allow to stand at room temperature for 30 mins.
6. Add an equal volume (600 uL) of reagent D (toluene). Vortex. Toluene will be the top layer. Retain it

and add an equal volume (600 uL) of reagent E (Na_2CO_3). Vortex.

7. Discard the top toluene layer and add 400 uL of reagent F (H_2O) and 1.0 mL of reagent G (NaOH) to the bottom layer. Vortex.

8. Centrifuge at least 1 minute in a tabletop centrifuge and read absorbance at 416 nm. Avoid any toluene which might be present by removing the sample below this layer with a Pasteur pipette.

Appendix X

Discontinuous Polyacrylamide Gel Electrophoresis

Stock solutions:

A. Running gel buffer

Tris base: 22.71 g
dH_2O: 75 mL
Adjust pH to 8.8 with HCl
Add dH_2O to a total volume of 100 mL

B. Stacking gel buffer

Tris base: 7.69 g
dH_2O: 75 mL
Adjust pH to 6.8 with HCl
Add dH_2O to a total volume of 100 mL

C. Acrylamide solution

Acrylamide: 25 g
Bis acrylamide: 0.625 g
Add dH_2O to a total volume of 50 mL

D. Sodium dodecylsulfate (SDS) solution (10%)

SDS: 2.5 g
dH_2O: 25 mL

E. Sample buffer

Solution B: 4.0 mL
Sucrose: 2 g
SDS: 0.4 g
Bromphenol blue: 5 mg
Mercaptoethanol: 1.0 mL
Add dH_2O to a total volume of 20 mL

F. Running buffer

Glycine: 14.4 g/L
Tris base: 3.0 g/L
SDS: 1.0 g/L

Preparing the Running Gel:

1. Assemble the gel electrophoresis apparatus.

2. For 30 mL of a 15% running gel, mix the following

solutions:

a. Solution A: 6.0 mL
b. Solution C: 9.0 mL
c. Solution D: 0.3 mL
d. dH_2O: 14.7 mL

3. Just prior to pouring the gel add 0.04 mL of a freshly prepared 10% ammonium persulfate solution and 0.02 mL of TEMED to the 25 mL of gel solution and mix.
4. Pour into the gel apparatus using a Pasteur pipette. Be careful not to introduce bubbles.
5. Using a glass syringe carefully overlay the gel solution with about 1.5 mL of dH_2O until the water covers the entire surface of the gel.
6. Allow the gel to polymerize, preferably overnight.

Preparing the Stacking Gel:

1. For 10 mL of a 4% stacking gel mix the following solutions:

a. Solution B: 2 mL
b. Solution C: 0.8 mL
c. Solution D: 0.1 mL
d. dH_2O: 7.1 mL

2. Remove the water from on top of the running gel and blot dry with a paper towel.
3. Just prior to pouring the stacking gel and 0.05 mL of a freshly prepared 10% ammonium persulfate solution and 0.02 mL of TEMED and mix.
4. Pour the stacking gel using a Pasteur pipette and be careful to avoid bubbles.
5. Insert the well-former, slowly and at a slight angle to

avoid trapping air bubbles beneath the wells.

6. Allow to polymerize.

Preparing the samples:

1. Add appropriate volume of sample to small tapered tube and lyophilize.
2. Add 25 uL of solution E and boil in a water bath for 5 min.
3. Fill the upper gel reservoir with running buffer (solution F) and carefully remove the well former.

Running the gel:

1. Using a syringe, load one sample in each well. Fill the lower reservoir with running buffer and assemble electrical connections.
2. Run at a constant current of about 20 ma. Electrophoresis is complete when the blue bromphenol dye reaches the bottom of the gel (about 4 hours).
3. Turn off the power, empty buffer reservoirs and carefully disassemble apparatus and remove the gel.
4. Stain the gel for appropriate macromolecules.

Note:

For sodium deoxycholate (DOC) gels the SDS is omitted and 0.5% DOC is used in the stacking and running gels and 0.25% DOC is used in the running buffer. The sample buffer contains 175mM Trizma base, 10% glycerine, and 0.25% DOC, pH 6.8. The gels are run at 18ma of constant current through the stacking gel and 25ma of constant current through the

running gel. The running of the sample should be preceded by pre-electrophoresis. Add a small amount of sample buffer to two wells at different ends of the gel and run at 20 ma until the dye has passed through the gel. Discard the running buffer and wipe the electrodes clean. Add fresh running buffer and run as described above. The gel should be used immediately after pre-electrophoresis.

Appendix XI

Silver Staining Prodecure for Lipopolysaccharides

Reagents:

A. Fixing solution:

40% ethanol, 5% glacial acetic acid, 55% deionized water

B. Oxidation solution: (prepare just before use)

1.05 g $NaIO_4$ (sodium metaperiodate), 150 mL fixing solution

C. Silver stain: (prepare just before use)

115 mL dH_2O, 28 mL 0.1M NaOH, 5 mL 20% AgNO3, and 1 mL of fresh conc. NH_4OH. The brown color should disappear with stirring after the ammonium hydroxide is added. If not add NH_4OH drop-wise until the brown precipitate just disappears.

D. Developer solution: (prepare just before use)

0.05 g citric acid, 0.5 mL of 37% formaldehyde, 0.5 L deionized water.

Procedure:

1. Place the gel in fixing solution and allow to shake slowly overnight. Change the fixing solution the next morning and afternoon. Again, allow to shake slowly overnight.
2. Place the gel in the oxidation solution for 5 minutes with shaking.
3. Remove the solution from the gel and wash with deioniz-

ed water with shaking 3 times, 15 min. each wash.

4. Place the gel in the silver stain for 10 minutes with shaking.
5. Remove the solution from the gel and wash with deionized water with shaking 3 times, 15 min. each wash.
6. Place the gel in the developer solution and allow bands to develop to desired darkness.
7. Remove the solution from the gel and wash with deionized water with shaking 3 times, 10 min. each wash. You may store the gel, wrapped in Saran wrap, at 4°C.

Appendix XII

Immunoblot Procedure

Reagents:

A. Transfer buffer:

25mM tris/187mM glycine prepared in 20% methanol

B. TBS solution with gelatin:

50 mM tris/200 mM NaCl , pH 7.4 with HCl, containing 0.5% gelatin

C. R. trifolii ANU843 antisera

D. TBS solution without gelatin:

50 mM tris/200 mM NaCl, pH 7.4 with HCl

E. Peroxidase conjugated antirabbit goat antisera (Sigma Chemical Co.)

F. Peroxide solution: (made fresh)

10 mL of 3 mg/mL 4-chloro-1-naphthol in methanol
40 mL of TBS
20 uL of 30% H_2O_2

Procedure:

1. Using a Hoeffer Transphor unit and transfer buffer, electroblot the LPSs from a slab gel onto nitrocellulose paper. Set the voltage at 80V for 1 hour.
2. Remove the nitrocellulose paper, rinse in deionized water, and allow to dry.
3. Place the paper in 100 mL of TBS solution with 0.5% gelatin.
4. After 30 min., add 1 mL on ANU843 antisera to the solution and allow to shake slowly for 2.5 hours.
5. Remove the antisera solution, replace with TBS, and

allow to shake for 10 min. Repeat two times.

6. Remove final TBS rinse and add 100 mL of TBS containing 0.5% gelatin and 0.1 mL of reagent E. Allow to shake slowly overnight at room temperature.
7. Remove this solution, replace with TBS, and allow to shake for 10 min. Repeat two times.
8. Develop using reagent F.
9. Remove the paper, rinsed briefly in deionized water, and allow to dry.

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